DELIVERY OF SUPEROXIDE DISMUTASE TO NEURONAL CELLS

The present invention relates to compositions and methods for delivery of superoxide dismutase (SOD) to neuronal cells, and in particular for delivery of SOD to mitochondria in those cells. The present invention also provides methods of making the constructs.

A number of nervous system disorders would benefit considerably from rapid intervention with several therapeutic agents. Examples of such disorders are global, focal or spinal cord ischaemia induced by stroke or injury. Neurones injured by trauma or ischaemia produce elevated levels of transmitter substances which result in high levels of reactive oxygen species. These reactive oxygen species, in high concentrations are toxic to both the neurones and the surrounding cells which potentiates and amplifies the damage process. Rapid therapeutic intervention with agents to reduce oxidative stress in cases of neuronal injury caused by stroke or trauma could therefore significantly limit this secondary damage process. One such potential therapeutic agent is superoxide dismutase which neutralises the harmful effects of the superoxide radicals by converting them to hydrogen peroxide and oxygen.

Francis and co-workers demonstrated that post-ischaemic infusion of Copper/Zinc superoxide dismutase (Cu/Zn-SOD) reduces cerebral infarction following ischaemia/reperfusion in rats (Experimental Neurology (1997) 146, 435-443) through the reduction of damaging free-radical oxygen. Lim et al. have shown than administration of Cu/Zn-SOD attenuates the level of reperfusion injury following spinal cord ischaemia in dogs (Ann. Thorac. Surg. (1986) 42, 282-286). Cuevas et al. have similarly demonstrated protective effects of SOD, both on neurological recovery and spinal infarction, in ischaemic reperfusion injury of the rabbit spinal cord (Acta Anat. (1990) 137, 303-310. A major problem in the use of such therapies is the maintenance of useful concentration of the active agent at the site

of trauma. Enzymes such as Cu/Zn-SOD are rapidly cleared from the systemic circulation; in the case of the latter enzyme the $t_{1/2}$ in rat is 4-8 minutes. A number of strategies have been employed to overcome these difficulties. Matsumiaya et al. (Stroke (1991) 22, 1193-1200) conjugated Cu/Zn-SOD to polyethylene glycol to increase it half-life in the blood. Francis and co-workers (Experimental Neurology (1997) 146, 435-443) describe the neuronal delivery of Cn/Zn-SOD by fusion of the enzyme to the binding domain of tetanus toxin. None of these strategies, however, are particularly efficient at delivering the enzyme to the intracellular neuronal compartments where the enzyme can be effective.

In most eukaryotic species, two intracellular forms of superoxide dismutase exist: the Cu/Zn-SOD which is located within the cytoplasmic and nuclear compartments and manganese superoxide dismutase (Mn-SOD) which is located within the mitochondrial matrix. Human Mn-SOD is a tetrameric enzyme and is larger than the dimeric Cu/Zn-SOD. Several studies have shown that decreased Mn-SOD may be associated with one or more chronic diseases such as ovarian cancer (Nishida *et al.* (1995) Oncology Reports, 2,643-646) and diabetes (L'Abbe *et al.,* (1994) Proc Soc Exp Biol Med, 207, 206-274). In addition, mice in which the Mn-SOD gene has been knocked out exhibit several novel pathogenic phenotypes including severe anaemia, degeneration of neurones in the basal ganglia and brainstem, and progressive motor disturbances characterised by weakness and rapid fatigue (Lebovitz *et al.,* (1996) Proc Natl Acad Sci USA, 93, 9782-9787). In addition these mice showed extensive damage to the neuronal mitochondria. Overexpression of Mn-SOD in cell lines and transgenic mice showed that damage and apoptosis of neurones under oxidative stress was markedly reduced (Keller *et al.*, (1998) Journal of Neuroscience, 18, 687-697). Mitochondrial damage was also reduced. These data showed that superoxide accumulation and subsequent mitochondrial damage play key rol s in neuronal death induced by trauma both *in vitro* and *in vivo*. Delivery of agents which reduce the level of

oxidative stress to neuronal cells may therefore reduce neuronal cell death and afford considerable therapeutic benefits.

Mn-SODs of bacterial origin such as that from *Bacillus stearothermophilus* or *B. caldotenax* consist of two subunits and are smaller than the human isoform which is a tetramer. *B. stearothermophilus* and *B. caldotenax* Mn-SOD also have considerably lower immunogenicity than the human isoform which is an advantage for continued therapeutic use. As enzymes for therapeutic applications, however, they suffer from similar drawbacks to other SODs in that very little of the administered enzyme is retained within the tissues where it would be therapeutically beneficial.

The botulinum neurotoxins are a family of seven structurally similar, yet antigenically different, protein toxins whose primary site of action is the neuromuscular junction where they block the release of the transmitter acetylcholine. The action of these toxins on the peripheral nervous system of man and animals results in the syndrome botulism, which is characterised by widespread flaccid muscular paralysis (Shone (1986) in 'Natural Toxicants in Foods', Editor D. Watson, Ellis Harwood, UK). Each of the botulinum neurotoxins consist of two disulphide-linked subunits; a 100 kDa heavy subunit which plays a role in the initial binding and internalisation of the neurotoxin into the nerve ending (Dolly et. al. (1984) Nature, 307, 457-460) and a 50 kDa light subunit which acts intracellularly to block the exocytosis process (McInnes and Dolly (1990) Febs Lett., 261, 323-326; de Paiva and Dolly (1990) Febs Lett., 277, 171-174). Thus it is the heavy chains of the botulinum neurotoxins that impart their remarkable neuronal specificity.

Tetanus toxin is structurally very similar to botulinum neurotoxins but its primary site of action is the central nervous system where it blocks the release of inhibitory neurotransmitters from central synaps s (Renshaw cells). As described for th botulinum toxins above, it is domains within

the heavy chain of tetanus toxin that bind to receptors on neuronal cells.

The binding and internalisation (translocation) functions of the clostridial neurotoxin (tetanus and botulinum) heavy chains can be assigned to at least two domains within their structures. The initial binding step is energy-independent and appears to be mediated by one or more domains within the H_C fragment of the neurotoxin (C-terminal fragment of approximately 50kDa) (Shone *et al.* (1985), Eur. J. Biochem., 151, 75-82) while the translocation step is energy-dependent and appears to be mediated by one or more domains within the H_N fragment of the neurotoxin (N-terminal fragment of approximately 50kDa).

Isolated heavy chains are non-toxic compared to the native neurotoxins and yet retain the high affinity binding for neuronal cells. Tetanus and the botulinum neurotoxins from most of the seven serotypes, together with their derived heavy chains, have been shown to bind a wide variety of neuronal cell types with high affinities in the nM range (e.g botulinum type B neurotoxin; Evans *et al.* (1986) Eur. J. Biochem. 154, 409-416).

Another key characteristic of the binding of these neurotoxins is that tetanus, botulinum A, B, C_1, D, E and F neurotoxins all appear to recognise distinct receptor populations, and collectively the clostridial neurotoxin heavy chains provide high affinity binding ligands that recognise a whole family of receptors that are specific to neuronal cells.

However, whilst it is known to provide a fusion of a SOD with a neurotoxin heavy chain, this fusion has been found to be ineffective for delivery of SOD to neuronal cells and inactive in *in vitro* assays for potential therapeutic activity.

It is an object of the invention to provide compositions and methods for delivery of SOD to neuronal cells. A further object is to provide

compositions and methods for treatment or at least amelioration of conditions such as ischemic stroke.

Accordingly, the present invention provides a construct for delivering superoxide dismutase (SOD) to neuronal cells. In one aspect of the invention the construct consists of a SOD which has been combined with various functional protein domains to effect efficient targeting to the mitochondria within neuronal cells. The construct of specific embodiments of the invention, described in further detail below, contains the following elements:-

- a SOD which contains a leader sequence for targeting SOD to the mitochondria;
- a dimeric SOD which has low immunogenicity and high stability;
- a SOD which is linked to a domain that effects translocation across lipid membranes;
- a linkage between the SOD and the translocation domain that is cleaved within the neuronal cytosol; and
- a domain which selectively targets the construct to neuronal cells.

A first aspect of the invention thus provides a composition for delivery of superoxide dismutase (SOD) to neuronal cells, comprising:-

SOD; linked by a cleavable linker to
a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the SOD of the composition into the neuronal cell.

The linker is cleavable and thus, in use, after translocation of the SOD into the cell, the linker is cleaved to release SOD from the neuronal cell targeting domain. A suitable linker is a disulphide bridge between cysteine residues, one residue on the SOD and one residue on the neuronal cell targeting component, for example on the second domain. Another example of a linker is a site for a protease found in neuronal cells. In this way, the

linker is such that it is cleaved within the cell, separating the SOD from the other translocated portions of the composition.

By SOD is meant a sequence that has superoxide dismutase activity, and may also comprise a further sequence or sequences conferring additional properties on that portion of the constructs. For example, the SOD optionally also includes a sequence targeting the SOD to mitochondria in a neuronal cell.

The SOD may be a hybrid of Mn-SOD and a sequence targeting the hybrid to mitochondria. The SOD may be of bacterial or human origin, or a derivative thereof, and may be comprised of sequences from more than one origin, provided that it has superoxide dismutase activity.

The first domain may suitably be selected from (a) neuronal cell binding domains of clostridial toxins; and (b) fragments, variants and derivatives of the domains in (a) that substantially retain the neuronal cell binding activity of the domains of (a). The second domain is suitably selected from (a) domains of clostridial neurotoxins that translocate polypeptide sequences into cells, and (b) fragments, variants and derivatives of the domains of (a) that substantially retain the translocating activity of the domains of (a).

In an embodiment of the invention a construct comprises SOD linked by a disulphide bridge to a neuronal cell targetting component comprising a first domain that binds to a neuronal cell and a second domain that translocates the SOD into the neuronal cell. This construct is made recombinantly as a single polypeptide having a cysteine residue on the SOD which forms a disulphide bridge with a cysteine residue on the second domain. The SOD is covalently linked, initially, to the second domain. Following expression of this single polypeptide SOD is cleaved from the second domain leaving the SOD linked only by the disulphide bridge to the rest of the construct.

A second aspect of the invention provides a pharmaceutical composition for treatment of oxidative damage to neuronal cells comprising a composition according to the invention with a pharmaceutically acceptable carrier. This composition may be used to deliver SOD to a neuronal cell, for example by administration of the composition by injection.

A third aspect of the invention provides a method of preparing a composition according to the invention, comprising chemically linking SOD, linker and neuronal cell targeting components. The SOD preferably is free of cysteine residues and the method preferably comprises treating the SOD with a cross-linker which will form a disulphide bridge with a cysteine residue on the neuronal cell targeting domain.

In a further embodiment of the third aspect of the invention, there is provided a method of making a composition according to the invention comprising expressing a DNA that codes for a polypeptide having SOD, a linker, a neuronal cell targeting component. The polypeptide may further comprise a purification sequence and the method may further comprise purifying the polypeptide using this sequence and then cleaving the polypeptide to remove the purification sequence to leave SOD, the linker and the neuronal cell targeting component.

By virtue of the combination of properties defined above, constructs of the invention are surprisingly efficient at transporting SOD to the mitochondria within neuronal cells. The ability of the superoxide dismutase to be translocated into the cytosol by virtue of the 'translocation domain' within the construct and the cleavage of the enzyme from the latter domain within the cell is key to this targeting efficiency. As such the construct of the invention has considerable therapeutic value in treating neuronal diseases which results from oxidative stress and has several advantages over previously described SOD formulations. Mitochondria within cells containing high levels of superoxide radicals are particularly sensitive to

damage and the ability of the construct of the invention to target the SOD to these organelles offers considerable advantage in that the enzyme can act to remove the superoxide radicals where it is most required.

The construct of the invention may be used clinically in a variety of neuronal diseases which are caused or augmented by oxidative stress. Such conditions include ischaemic stoke, Parkinson's disease, Huntington's disease and motor neurone diseases. In the case of ischaemia/reperfusion injury caused by stroke or trauma, delivery of the construct of the invention to neurones of the hippocampus may afford considerable therapeutic benefits by reducing neuronal damage and death. Other neuronal diseases where the underlying cause is oxidative stress would also benefit from the therapeutic effects of the construct of the invention.

In a preferred aspect of the invention, the SOD is a dimeric, manganese superoxide dismutase (Mn-SOD) which is of bacterial origin and has low immunogenicity and high stability.

The use of a bacterial Mn-SOD in constructs has a number of advantages compared to the use of the human Mn-SOD isoform:-

- the low immunogenicity of the bacterial Mn-SOD is advantageous where repeated administration of the construct is required, in which cases the induction of adverse host immune responses is reduced; and

- the smaller size of constructs based on the dimeric bacterial Mn-SOD compared to human Mn-SOD (which is a tetramer) both reduces the likelihood of adverse immune responses and increases the rate of diffusion of the construct to its target tissue.

In exercise of an example of the invention, a bacterial Mn-SOD of low

immunogenicity is derived from either *B. stearothermophilus* (sequence as reported by Brock and Walker (1980) Biochemistry, 19, 2873-2882) or *B. caldotenax* (gene and amino acid sequence as defined by Chambers *et al.*, (1992) FEMS Microbiology Letters, 91, 277-284) to which a mitochondrial leader sequence has been fused to the N-terminus of the protein by recombinant technology. This Mn-SOD-leader hybrid is linked by a disulphide bridge to a translocation domain derived from a bacterial protein toxin, such as botulinum neurotoxin. The translocation domain, in turn is fused to a receptor binding domain derived from a clostridial neurotoxin (botulinum or tetanus). The construct is produced initially as a single polypeptide by recombinant technology and subsequently converted to the construct of the invention by selective cleavage with a proteolytic enzyme. To produce the construct of the invention, a loop motif containing a unique protease site (e.g amino acid sequences specifically cleaved by proteases such as factor Xa, enterokinase, thrombin) and a cysteine residue is introduced between the C-terminus of the Mn-SOD and the N-terminus of the translocation domain such that a disulphide bridge is formed between the Mn-SOD and the translocation domain. Subsequent cleavage of the protease site generates the active construct. The final construct, when analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis in the presence of a reducing agent (e.g. dithiothreitol), dissociates into two major bands, one corresponding to the superoxide dismutase enzyme and a second corresponding to a polypeptide which contains the neuronal binding and translocation domains. In the absence of a reducing agent this dissociation does not occur and the complex is observed as a single major band on the gels.

Modification of Mn-SOD from *B. stearothermophilus* by addition of a mitochondrial targeting sequence offers several advantages over the use of human Mn-SOD which contains its own mitochondrial leader sequence. Firstly, the *B. stearothermophilus* Mn-SOD has a high thermal stability and low immunogenicity which allows administration of several doses of the

enzyme without provoking an immune response from the host that would reduces its efficacy. Secondly, the *B. stearothermophilus* Mn-SOD is a small dimeric enzyme unlike the human Mn-SOD which is a tetramer. Recombinant constructs containing the latter enzyme would therefore have to be considerably larger and more complex in their structure.

In an embodiment of the invention, a DNA encoding a construct of the invention is made up by fusion of following DNA fragments commencing at the 5'end of the gene:-

an oligonucleotide encoding a modified human mitochondrial leader sequence (amino acid sequence: MLSRAVCGTSRQLAPALGYLGSRQ (SEQ ID NO:10) or MLSRAVSGTSRQLAPALGYLGSRQ (SEQ ID NO:11);

an oligonucleotide encoding Mn-SOD from *B. stearothermophilus* (coding for the amino acid sequence as defined in Brock and Walker (1980) Biochemistry, 19, 2873-2882);

an oligonucleotide encoding a linker peptide which contains the thrombin protease cleavage site and a cysteine residue for disulphide bridge formation (peptide sequence: CGLVPAGSGP);

an oligonucleotide encoding a translocation domain derived from a botulinum neurotoxin (e.g. a DNA fragment coding for amino acid residues 449-871 of botulinum type A neurotoxin, or a DNA fragment coding for amino acid residues 441-858 of botulinum type B neurotoxin, or a DNA fragment coding for amino acid residues 440-864 of botulinum type F neurotoxin); and

an oligonucleotide encoding the receptor binding domain of a botulinum neurotoxin or tetanus neurotoxin (e.g. a DNA fragment

coding for amino acid residues 872-1296 of botulinum type A neurotoxin, or a DNA fragment coding for amino acid residues 859-1291 of botulinum type B neurotoxin, or a DNA fragment coding for amino acid residues 865-1278 of botulinum type F neurotoxin, or a DNA fragment coding for amino acid residues 880-1315 of tetanus neurotoxin).

The above DNA fragments may be obtained and constructed by standard recombinant DNA methods. Expression and purification of the assembled construct may be obtained with a variety of suitable expression hosts, e.g. *Escherichia coli*, *Bacillus subtilis*.

The translocation domain and neuronal binding domain of the construct may also be derived from combination of different clostridial neurotoxins. For example, the construct of the invention may contain a translocation domain derived from botulinum type F neurotoxin and a binding domain derived from botulinum type A neurotoxin.

A construct of the invention may be produced using protein chemistry techniques. Mn-SOD derived from *B. stearothermophilus* to which a mitoch ondrial leader sequence has been fused to the N-terminus of the protein by recombinant technology is modified with a heterobifunctional cross-linking reagent such as N-succinimidyl 3-[2-pyridyldithio] propionate (SPDP). The chemically modified enzyme is then combined to a cell targetting domain which contains the binding and translocation functional domains. The latter may be produced by recombinant technology or purified from the neurotoxins of *Clostridium botulinum* or *Clostridium tetani* by established methods. Chemical coupling of the SPDP-treated Mn-SOD may be accomplished using a free cysteine residue on the polypeptide containing the binding and translocation domains to give a construct of the invention.

Constructs of the invention may be introduced into either neuronal or non-neuronal tissue using methods known in the art. By subsequent specific binding to neuronal cell tissue, the targeted construct will exert its therapeutic effects. Alternatively, the construct may be injected near a site requiring therapeutic intervention, e.g. intrathecal or intracranial injection close to a site of trauma or disease.

The construct of the invention may also be administered with other agents which enhance its delivery to its target tissue. An example of such an agent is one which assists the passage of the construct of the invention through the blood-brain barrier to the central nervous system. The construct of the invention may also be administered in formulations with other therapeutic agents or drugs.

The dosage required for the construct of the invention will depend upon the application and could vary between 1μg/kg to 100mg/kg of body weight. The construct of the invention may be produced as a suspension, emulsion, solution or as a freeze dried powder depending on the application and properties of the release vehicle and its therapeutic contents. The construct of the invention may be resuspended or diluted in a variety of pharmaceutically acceptable liquids depending on the application.

"Clostridial neurotoxin" means a neurotoxin corresponding to tetanus neurotoxin or one of the seven botulinum neurotoxin serotypes (type A, B, C_1, D, E, F or G).

"Bind" in relation to the clostridial binding fragments, means the interaction between the clostridial fragment and one or more cell surface receptors or markers which results in localisation of the binding fragment or construct in the vicinity of the cell.

"Binding domain" of botulinum or tetanus neurotoxins means a domain of

the toxin which retains the property of being abl to bind the receptors on neuronal cells in a similar manner to the intact n urotoxin and encompasses native domains and fragments, variants and derivatives that retain this binding function. This property of the binding domain can be assessed in competitive binding assays. In such assays, radiolabelled neurotoxin (e.g botulinum type A neurotoxin) is contacted with neuronal cells in the presence of various concentrations of non-radiolabelled fragment representing the 'binding domain' of the neurotoxin. The ligand mixture is incubated with the cells, at low temperature (0-3°C) to prevent ligand internalisation, during which competition between the radiolabelled neurotoxin and non-labelled 'binding domain' fragment may occur. In such assays when the unlabelled ligand used is binding domain of botulinum type A neurotoxin (residues 872-1296), the radiolabelled botulinum type A neurotoxin will be displaced from the neuronal cell receptors as the concentration of its non-labelled 'binding domain' is increased. The competition curve obtained in this case will therefore be representative of the behaviour of a 'binding domain' fragment being a able to bind the receptors on neuronal cells in a similar manner to the intact neurotoxin. This property of the binding domain may be used to identify other suitable protein domains which have the desired binding properties. Examples of binding domains derived from clostridial neurotoxins are as follows:-

Botulinum type A neurotoxin	– amino acid residues (872 - 1296)
Botulinum type B neurotoxin	– amino acid residues (859 - 1291)
Botulinum type C neurotoxin	– amino acid residues (867 - 1291)
Botulinum type D neurotoxin	– amino acid residues (863 - 1276)
Botulinum type E neurotoxin	– amino acid residues (846 - 1252)
Botulinum type F neurotoxin	– amino acid residues (865 - 1278)
Botulinum type G neurotoxin	– amino acid residues (864 - 1297)
Tetanus neurotoxin	– amino acid residues (880 - 1315)

"Translocation domain" means a domain or fragment of a protein which ffects transport of itself and/or other proteins and substances across a

membrane or lipid bilayer and encompasses native domains and fragments, variants and derivatives that retain this binding function. The latter membrane may be that of an endosome where translocation will occur during the process of receptor-mediated endocytosis. Translocation domains can frequently be identified by the property of being able to form measurable pores in lipid membranes at low pH (Shone *et al.* (1987) Eur J. Biochem. 167, 175-180). The latter property of translocation domains may thus be used to identify other protein domains which could function as the translocation domain within the construct of the invention. Examples of translocation domains derived from bacterial neurotoxins are as follows:-

Botulinum type A neurotoxin	– amino acid residues (449 - 871)
Botulinum type B neurotoxin	– amino acid residues (441 - 858)
Botulinum type C neurotoxin	– amino acid residues (442 - 866)
Botulinum type D neurotoxin	– amino acid residues (446 - 862)
Botulinum type E neurotoxin	– amino acid residues (423 - 845)
Botulinum type F neurotoxin	– amino acid residues (440 - 864)
Botulinum type G neurotoxin	– amino acid residues (442 - 863)
Tetanus neurotoxin	– amino acid residues (458 - 879)

"Translocation" in relation to translocation domain, means the internalisation events which occur after modified clostridial binding fragments bind to the cell surface. These events lead to the transport of substances into the cytosol of neuronal cells.

"Unique protease site" means a protease site incorporated into the construct such that the molecule may be proteolysed at pre-determined sites by a selected protease. The specificity of these proteases is such that cleavage to other parts of the construct does not occur. Examples of unique protease sites are the amino acid sequences cleaved by proteases such as: thrombin, factor Xa, enterokinase.

A fourth aspect of th invention provides a composition for delivery of a

therapeutic agent to neuronal cells, comprising:-

the therapeutic agent; linked by a cleavable linker to

a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the therapeutic agent of the composition into the neuronal cell.

Thus, in use, after translocation of the therapeutic agent into the cell, the linker is cleaved to release the therapeutic agent from the neuronal cell targeting domain. Other optional and preferred embodiments of the fourth aspect of the invention are as for the first-third aspects of the invention.

A fifth aspeect of the invention provides a polypeptide comprising a bacterial SOD, or derivative thereof, and a sequence for targeting the polypeptide to a mitochondria, such as a human mitochondria. The polypeptide may be chemically obtained by synthesis of otherwise or may be a fusion protein, obtained for example by expression of a nucleotide coding for the polypeptide.

The invention hence also provides, in a sixth aspect, a nucleotide encoding the polypeptide of the fifth aspect and in a seventh aspect a vector comprising the nucleotide of the sixth aspect. Also provided in an eigth aspect is a method of making a polypeptide according to the fifth aspect comprising expressing the nucleotide sequence of the sixth aspect. In a ninth aspect is provided a cell comprising the nucleotide sequence of the sixth aspect or the vector of the seventh.

There now follows description of specific embodiments of the invention illustrated by drawings in which:-

Fig. 1 shows schematic xamples of novel Mn-SODs derived from *B. stearothermophilus* and *B. caldotenax*. Two examples of mitochondrial leader sequences are shown. In one example, a cysteine residue at position

7 has been mutated to a serine residue. This change enables the production of the construct of the invention without the formation of disulphide bridges in undesirable positions;

Figure 2 shows schematic examples of novel Mn-SOD fusion proteins showing the use of peptides and proteins to facilitate purification of the enzyme from the production strain. Various protein and peptide tags (such as histidine-6, S-peptide, maltose-binding protein, calmodulin-binding protein) may be fused to the Mn-SOD to allow rapid purification by affinity chromatography methods. Unique protease sites are incorporated between the purification tag and the Mn-SOD to enable removal of the tag after purification. Protein and peptide tags may be removed by treatment of the fusion protein with the relevant specific protease (e.g. factor Xa, thrombin, enterokinase);

Fig. 3 shows a recombinant Mn-SOD construct of the invention. From the N-terminus of the protein, the construct consists of the following components:- (1) a mitochondrial leader (targeting) sequence, (2) a Mn-superoxide dismutase, (3) a loop which contains a unique protease site and which allows disulphide bridge formation, (4) a translocation domain, (5) a neuronal targeting domain. The construct is produced as a single polypeptide; subsequent cleavage with a protease specific for the 'unique protease site' contained within the loop region generates the di-chain construct. Purification tags could added to the constructs as exemplified in Figure 3;

Fig. 4 shows the production of a Mn-SOD construct by chemical methods. The method uses a recombinant Mn-SOD, purified as described in Example 1 and coupled to a polypeptide containing the translocation and binding domains as d scribed in Example 4; and

Fig. 5 shows the results of an example to demonstrate the protective

effects of a construct of the invention on NG108 cells subjected to oxidative stress by the addition of 50μM duroquinone for four hours.

The application is also accompanied by a sequence listing in which:-

SEQ ID NO: 1 shows the amino acid sequence of Mn-SOD from *B. caldotenax*;

SEQ ID NO: 2 shows the amino acid sequence of Mn-SOD from *B. stearothermophilus*;

SEQ ID NO: 3 shows the amino acid sequence of a construct of the invention comprising Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype A;

SEQ ID NO: 4 shows the amino acid sequence of a construct of the invention comprising Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype B;

SEQ ID NO: 5 shows the amino acid sequence of a construct of the invention comprising Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype F;

SEQ ID NO: 6 shows the amino acid sequence of a construct of the invention comprising a mitochondrial leader sequence from human Mn-SOD, Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype A;

SEQ ID NO: 7 shows the amino acid sequence of a construct of the invention comprising a mitochondrial leader sequence from human Mn-SOD, Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype B;

SEQ ID NO: 8 shows the amino acid sequence of a construct of the invention comprising a mitochondrial leader sequence from human Mn-SOD, Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype F; and

SEQ ID NO: 9 shows the amino acid sequence for a polypeptide comprising a mitochondrial leader sequence from human Mn-SOD and Mn-SOD from *B. stearothermophilus*;

SEQ ID NO: 10 shows the amino acid sequence of a modified human mitochondrial leader sequence; and

SEQ ID NO: 11 shows an amino acid sequence of a modified human mitochondrial leader sequence.

Example 1.

Production and purification of novel *B. stearothermophilus* Mn-SOD containing a mitochondrial leader sequence.

Standard molecular biology protocols were used for all genetic manipulations (*eg.* Sambrook *et al.* 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). A synthetic gene encoding the mitochondrial targeting sequence (amino acids 1-27) of the human Mn-SOD gene was cloned as

an *Nde*I - *Bam* HI fragment into an expression vector so that the transcriptional start corresponds to the ATG codon within the *Nde*I site. The Mn-SOD gene from *B. stearothermophilus* or *B. caldotenax* was amplified using PCR to give a *Bg*III site corresponding to the leucine amino acid at position 5 at the 5' end, and a *Bam*HI site outside the stop codon at the 3' end. This *Bs*III-*Bam*HI fragment was cloned into the expression vector carrying the mitochondrial targeting sequence (digested *Bam*HI) to generate "in-frame" gene fusions. In addition to the wild type mitochondrial targeting sequence, a variant was constructed in which the cysteine at position 7 was changed to serine

The recombinant Mn-SOD expressed in pET28a were produced with amino-terminal histidine (6 His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Ni^{2+} charged column or an anti-T7 immunoaffinity column (Smith *et al.* 1988, *Journal of Biological Chemistry*, 263: 7211-7215). Incorporation of a factor Xa protease cleavage between the peptide tag and the expressed Mn-SOD allowed this to be removed after purification. Briefly, cultures of *E.coli* BL21 (DE3) pET28a-Mn-SOD were grown in Terrific broth-kanamycin (30 $\mu g ml^{-1}$) to an OD_{600} nm of 2.0, and protein expression was induced by the addition of 500μM IPTG for approximately 2 h. Cells were lysed by freeze/thaw followed by sonication, lysates cleared by centrifugation and supernatants loaded onto an anion exchange column (MonoQ™ column on a Fast Protein Liquid Chromatography system; Pharmacia Biotech, Uppsala, Sweden). Eluted recombinant Mn-SOD was then desalted and further purified by affinity chromatography on a chelating sepharose column charged with Ni^{2+} (Pharmacia Biotech, Uppsala, Sweden). After loading proteins onto the column and subsequent washing, the purified Mn-SOD was eluted with imidazole. All buffers used were as specified by the manufacturer.

A 'maltose binding protein' purification tag was also employed for the purification of some batches of Mn-SOD. The use of this system is

described in detail in New England Biolabs Instruction Manual "Protein Fusion and Purification System" (ver 3.02).

Other tags and protease cleavage site may also be incorporated into the sequence to facilitate purification of Mn-SOD as exemplified in Figure 2.

For purification of a novel *B. stearothermophilus* Mn-SOD which was not conjugated to protein purification tag, the following procedure was used. After harvesting, cells were broken by high pressure homogenisation crude extracts were clarified by centrifugation and batch purified on DE-23 cellulose. The fraction eluted with 0.4M NaCl contained the Mn-SOD. This fraction was then further purified by various chromatographic media using the following sequence:-

DEAE-Sepharose ion exchange chromatography at pH 8.0; elution of the Mn-SOD with a NaCl gradient;

hydroxylapatite chromatography at pH 6.8; elution of Mn-SOD with a phosphate gradient at pH 6.8;

ion exchange chromatography on Q-Sepharose at pH 7.5; elution with a NaCl gradient; and

gel filtration on Sephacryl S-200.

The purified Mn-SOD may be dialysed against Hepes buffer (0.1M, pH7.4) containing 0.15M NaCl and stored at –80°C.

Example 2.

Preparation and purification of a recombinant Mn-SOD construct of the invention.

Standard molecular biology protocols were used for all genetic manipulations (*eg.* Sambrook *et al.* 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Various DNA fragments of the construct were generated using Recursive PCR reactions (Prodromou & Pearl 1992, *Protein Engineering*, 5: 827-829) using self-priming oligonucleotides containing the desired sequence. For the expression of clostridial neurotoxin fragments the codon bias and GC/AT base ratio was adjusted for ease of expression in *E. coli*. Fragments were cloned sequentially into pLitmus 38 (New England Biolabs, Inc., Beverly, MA) to assemble the entire gene. Constructs for expression were sub-cloned into pET28b (Novagen Inc., Madison, WI) replacing the *Eco*R1-*Hin*dIII fragment. The ligation reactions were transformed into *E.coli* DH5α (Life Technologies Inc., Gaithersburg, MD). Plasmid DNA was amplified, purified and screened for the presence of the appropriate sequence (Ausubel *et al.* 1989, Current Protocols in Molecular Biology, John Wiley & Sons, New York). Gene constructions confirmed as possessing the correct sequences were then transformed into the expression host *E. coli* BL21 (DE3) (Studier & Moffatt 1986, *Journal of Molecular Biology*, 189: 113-130).

The recombinant constructs expressed in pET28 were produced with amino-terminal histidine (6 His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Ni^{2+} charged column or an anti-T7 immunoaffinity column (Smith *et al.* 1988, *Journal of Biological Chemistry*, 263: 7211-7215). Incorporation of a factor X protease cleavage between the peptide tags and the expressed Mn-SOD Constructs allowed these to be removed after purification. Briefly, cultures of *E.coli* BL21 (DE3) pET28-Mn-SOD Construct were grown in Terrific broth-kanamycin (30 μgml^{-1}) to an OD_{600} nm of 2.0, and protein expression was induced by the addition of 500μM IPTG for approximately 2 h. Cells were lysed by freeze/thaw followed by sonication, lysates cleared by centrifugation and supernatants loaded onto an anion exchange column (MonoQ™ column on

a Fast Protein Liquid Chromatography system; Pharmacia Biotech, Uppsala, Sweden). Eluted recombinant Mn-SOD Construct was then desalted and further purified by affinity chromatography on a chelating sepharose column charged with Ni^{2+} (Pharmacia Biotech, Uppsala, Sweden). After loading proteins onto the column and subsequent washing, the purified Construct was eluted with imidazole. All buffers used were as specified by the manufacturer.

A 'maltose binding protein' purification tag was also employed for the purification some batches of Mn-SOD Constructs. The use of this system is described in detail in New England Biolabs Instruction Manual "Protein Fusion and Purification System" (ver 3.02).

It would also be evident to anyone skilled in the art that other tags and protease cleavage site may also be incorporated into the sequence to facilitate purification as exemplified in Figure 3.

The amino sequences of several recombinant Mn-SOD constructs are shown in the sequence listing.

Example 3.

Preparation of botulinum heavy chains by chemical methods.

The various serotypes of the clostridial neurotoxins may be prepared and purified from various toxigenic strains of *Clostridium botulinum* and *Clostridium tetani* by methods employing standard protein purification techniques as described previously (Shone and Tranter 1995, Current Topics in Microbiology, 194, 143-160; Springer). Samples of botulinum neurotoxin (1mg/ml) are dialysed against a buffer containing 50mM Tris-HCl pH 8.0, 1M NaCl and 2.5M urea for at least 4 hours at 4°C and then made 100mM with dithiothreitol and incubated for 16h at 22°C. The cloudy

solution which contains precipitated light chain is then centrifuged at 15000 x g for 2 minutes and the supernatant fluid containing the heavy chain retained and dialysed against 50mM Hepes pH 7.5 containing 0.2M NaCl and 5mM dithiothreitol for at least 4 hours at 4°C. The dialysed heavy chain is centrifuged at 15000 x g for 2 minutes and the supernatant retained and dialysed thoroughly against 50mM Hepes pH 7.5 buffer containing 0.2M NaCl and stored at -70°C. The latter procedure yields heavy chain >95% pure with a free cysteine residue which can be used for chemical coupling purposes. Biological (binding) activity of the heavy chain may be assayed as described in Example 5.

The heavy chains of the botulinum neurotoxins may also be produced by chromatography on QAE Sephadex as described by the methods in Shone and Tranter (1995) (Current Topics in Microbiology, 194, 143-160; Springer).

Example 4

Production of Mn-SOD constructs by chemical methods

B. stearothermophilus Mn-SOD fused to a mitochondrial leader sequence was purified as described in Example 1. The Mn-SOD was chemically modified by treatment with a 3-5 molar excess of N-succinimidyl 3-[2-pyridyldithio] propionate (SPDP) in 0.05M Hepes buffer pH 7.0 containing 0.1M NaCl for 60 min at 22°C. The excess SPDP was removed by dialysis against the same buffer at 4°C for 16h. The substituted SOD was then mixed in a 1:2.5 molar ratio with heavy chain purified from *Clostridium botulinum* type A neurotoxin purified as described in Example 3 and incubated at 4°C for 16h. During the incubation period the Mn-SOD was conjugated to the botulinum heavy chain fragment by free sulphydryl groups (see Figure 4). After incubation, the Mn-SOD-construct was purified by gel filtration chromatography on Sephadex G200.

Constructs of the invention may also be formed by the above method using polypeptides containing the translocation and binding domains that have been produced by recombinant technology as outlined in Example 2.

Example 5.

Assay of the biological activity of constructs - demonstration of high affinity binding to neuronal cells.

Clostridial neurotoxins may be labelled with 125-iodine using chloramine-T and its binding to various cells assessed by standard methods such as described in Evans *et al.* 1986, Eur J. Biochem., 154, 409 or Wadsworth *et al.* 1990, Biochem. J. 268, 123). In these experiments the ability of Mn-SOD constructs to compete with native clostridial neurotoxins for receptors present on neuronal cells or brain synaptosomes was assessed. All binding experiments were carried out in binding buffers. For the botulinum neurotoxins this buffer consisted of: 50mM HEPES pH 7.0, 30mM NaCl, 0.25% sucrose, 0.25% bovine serum albumin. For tetanus toxin, the binding buffer was: 0.05M tris-acetate pH 6.0 containing 0.6% bovine serum albumin. In a typical binding experiment the radiolabelled clostridial neurotoxin was held at a fixed concentration of between 1-20nM. Reaction mixtures were prepared by mixing the radiolabelled toxin with various concentrations of unlabelled neurotoxin or construct. The reaction mixture were then added to neuronal cells or rat brain synaptosomes and then incubated at 0-3°C for 2hr. After this period the neuronal cells of synaptosomes were washed twice with binding ice-cold binding buffer and the amount of labelled clostridial neurotoxin bound to cells or synaptosomes was assessed by γ-counting. In an experiment using an Mn-SOD construct which contained the binding domain from botulinum type A neurotoxin, the construct was found to compete with ^{125}I-labelled botulinum type A neurotoxin for neuronal cell receptors in a similar manner to unlabelled native botulinum type A neurotoxin. These data showed that

the construct had retained binding properties of the native neurotoxin.

Example 6

Assay of the biological activity of constructs – measurement of the Mn-SOD activity.

Mn-SOD activity in samples and constructs was measured by a modification (Brehm *et al.* (1991) Appl. Microbiol. Biotechnol., 36,358-363) of the procedure described by McCord and Fridovich (J. Biol. Chem. (1969), 244, 6049-6055). Aliquots (20μl) of samples or constructs containing Mn-SOD were added to 1ml of 0.05M potassium phosphate buffer pH 7.5 containing 1 x 10^{-4} M EDTA, 2.5 x 10^{-5}M ferricytochrome C and 7 x 10^{-3}M sodium xanthine in a thermostatted cuvette at 30°C. Sufficient xanthine oxidase was added to produce a rate of reduction of the ferricytochrome C at 550nm of approx. 0.1 absorbance units/minute in the absence of Mn-SOD. Under these conditions the amount Mn-SOD that was required to reduce the rate of reduction of ferricytochrome C by 50% was defined as one unit of activity.

Using such assays the Mn-SOD activity within constructs was assessed.

Example 7

Demonstration of the targeting of Mn-SOD to the mitochondria of neuronal cells by constructs of the invention

Mn-SOD construct containing the translocation and targeting domains derived from botulinum type A neurotoxin was incubated at various concentrations (0.01-10μM final concentration) with a neuroblastoma cell line NG108. Incubations were carried out over a 6h period or overnight at 37°C. In some experiments, construct radiolabelled with ^{125}iodine was

used. After incubation with the construct cells, were removed from culture flasks by gentle scraping and centrifuged at 200 x g. Cells were then resuspended in breaking buffer (0.6M mannitol, 20mM hepes pH 7.4 and 1mM phenylmethylsulphonyl chloride) and homogenised in a Dounce homogeniser. The homogenate was centrifuged at 200 x g for 5min and then the supernatant fluid recovered and centrifuged at 8000 x g for 10min. The 200 x g pellet (nuclear fraction) were pooled and resuspended in phosphate buffered saline. The 8000 x g pellets (mitochondrial fraction) were also pooled and resuspended in phosphate buffered saline. The supernatant fluid was saved and used to represent the cytosolic fraction.

Analysis of the sub-cellular distribution of Mn-SOD was carried out by Western blot analysis and, where radiolabelled construct was used, by analysis of the ^{125}I-labelled construct components by *y*-counting and by autoradiography of cell fractions which had been separated by electrophoresis on SDS-polyacrylamide gels. For Western blot analysis, proteins in the cell fractions were separated by electrophoreses on SDS-polyacrylamide gels and then transferred to nitrocellulose membrane as described previously (Towbin *et al.* Proc.(1979) Natl. Acad. Sci. USA, 76, 4350). The presence of Mn-SOD in protein bands on nitrocellulose membranes was assessed by incubation with rabbit anti- Mn-SOD antibody followed by washing and incubation with anti-rabbit peroxidase conjugate. Addition of peroxidase substrates (3,3',5,5'- tetramethyl benzidine and H_2O_2) allowed visualisation and quantitation of the Mn-SOD in the various sub-cellular protein fractions. An enhanced chemiluminescence system (Amersham International) was also used in some experiments to increase the sensitivity.

Example 8.

Formulation of the Mn-SOD construct for clinical use.

In a formulation of the Mn-SOD construct for clinical use, recombinant Mn-SOD construct would be prepared under current Good Manufacturing Procedures. The construct would be transferred, by dialysis, to a solution to give the product stability during freeze-drying. Such a formulation may contain Mn-SOD construct (10 mg/ml) in 5mM HEPES buffer (pH 7.2), 50mM NaCl, 1% lactose. The solution, after sterile filtration, would be aliquotted, freeze-dried and stored under nitrogen at -20°C.

Example 9.

Use of an Mn-SOD construct to treat stroke.

In a typical case of a middle aged or elderly man diagnosed as suffering from stroke, treatment with an Mn-SOD construct would begin immediately, ideally within 6 hours of the stroke occurring. Doses of the Mn-SOD construct (e.g. 100mg) reconstituted in a sterile saline solution would be administered intravenously. Further doses of the construct would be administered daily for 5-10 days. Such a patient would be expected to display reduced levels of ischaemia/reperfusion damage as assessed by magnetic resonance imaging compared to a similarly affected patient receiving no treatment. Relative improvements to muscle strength and co-ordination (MRC motor score) would be expected to be observed over the subsequent 12 month period.

Example 10.

Method to Demonstrate Neuroprotection of Cells by MnSOD-Heavy Chain Conjugates

Neuroblastoma cell line NG108-15 (*Nature* (1998) 336:p185 were seeded at a density of $3x10^4$cells/ml in 96 well microtitre plates coated with poly-D-lysine. Plates were grown for 3 days at 37°C in a CO_2 incubator (5% CO_2

95% air). MnSOD or leader-MnSOD conjugates were prepared with purified heavy chain of botulinum neurotoxin serotype A (BoNT/A HC) as described. The concentration of SOD was estimated and the conjugate diluted to give the specified amount of conjugate in a total volume of 200μl serum free medium. Conjugate was added to wells in the presence or absence of 56mM KCl, 2mM $CaCl_2$. The cells were incubated with conjugate for 1 hour. The conjugate was replaced with either serum free medium or serum free medium containing 50μM duroquinone and incubated at 37°C for 4 hours in the CO_2 incubator to induce oxidative stress. The media was removed after 4 hours and replaced with the dye 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.25mg/ml in serum free medium and incubated for 2 hours (according to the method of Mattson, MP, *et al* (1995) *Methods in Cell Biology* 46:187-216) The conversion of MTT to formazan dye crystals has been shown to be related to mitochondrial respiratory chain activity (Musser, DA, and Oseroff, AR (1994) *Journal of Immunology* 59:621-626). MTT was removed and crystals solubilised with dimethylsulfoxide (DMSO). Absorbance at 570nm was measured using a Labsystems Multiskan Plus MkII spectrophotometer and the results shown in Figure 5.

Figure 5 shows that a construct of the invention was able to protect neuronal cells against the mitochondrial-focused oxidative stress produced by duroquinone.

Example 11.

Preparation and purification of recombinant MnSOD and leader sequence MnSOD

Standard molecular biology protocols were used for all genetic manipulations (Sambrook *et al* 1989, Molecular cloning; A laboratory manual. Second Edition, Cold Spring Harbor Laboratory Press, New York.).

The MnSOD gene from either *B. stearothermophilus* or *B.caldotenax* was amplified by PCR to engineer a *Bam*HI site (resulting in the replacement of nucleotides 1-15). A synthetic oligonucleotide corresponding to the mitochondrial leader sequence of human MnSOD (bases 1-81of the human gene) was subcloned into the *Bam*HI site to generate leader-MnSOD. PCR was used to add a Factor Xa cleavage site immediately adjacent to the methionine at the start of the leader sequence. Similarly a Factor Xa cleavage site was engineered immediately adjacent to the methionine at the start of the native MnSOD gene. Constructs were sequenced to confirm the presence of the correct sequence. Constructs for expression were subcloned into the expression vector pET28a (Novagen Inc, Madison, WI) as an *Eco*RI fragment and the orientation of the fragments checked. Clones with confirmed sequences were used to transform expression host *E.coli* BL21 (DE3) (Studier and Moffatt 1986 *Journal of Molecular Biology* **189**:113-130). Examples 2 and 3 above provide detailed methods.

The recombinant proteins expressed from pET28a contain amino-terminal histidine (6-His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Cu^{2+} charged metal chelate column or an anti-T7 immunoaffinity column. Incorporation of the Factor Xa site between the peptide tags and the start of either MnSOD or leader MnSOD allows the precise removal of the peptide tags after purification. Cultures of *E.coli* BL21(DE3) pET28a-MnSOD or BL21(DE3) pET28a-leader-MnSOD were grown in Terrific Broth containing 30μg/ml kanamycin and 0.5% (w/v) glucose to an OD_{600} of 2.0 and protein expression was induced with 500μM IPTG for 2 hours. Cells were lysed by sonication, cell debris pelleted by centrifugation and the supernatant loaded onto a metal chelate column charged with Cu^{2+} (Amersham-Pharmacia Biotech, Uppsala, Sweden). After loading proteins on the column and washing, proteins were eluted using imidazole. All buffers were used as specified by manufacturers. Factor Xa cleavage of the eluted protein was carried out according to manufacturers instructions.

The invention thus provides constructs and methods for delivery of SOD to neuronal cells.